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Review article

Mouse models of cytomegalovirus latency: overview

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Abstract

Background: The molecular regulation of viral latency and reactivation is a central unsolved issue in the understanding of cytomegalovirus (CMV) biology. Like human CMV (hCMV), murine CMV (mCMV) can establish a latent infection in cells of the myeloid lineage. Since mCMV genome remains present in various organs after its clearance from hematopoietic cells first in bone marrow and much later in blood, there must exist one or more widely distributed cell type(s) representing the cellular site(s) of enduring mCMV latency in host tissues. Endothelial cells and histiocytes are candidates, but the question is not yet settled. Another long debated problem appears to be solved: mCMV establishes true molecular latency rather than a low-level persistence of productive infection. This conclusion is based on two recent advances. First, on a highly improved assay of infectivity, and second, on very sensitive RT PCRs for detecting viral transcripts during latency. In essence, infectious virus and productive cycle transcripts, such as transcripts of early-phase gene *M55* (gB) and *ie3* transcripts specifying the essential transactivator protein IE3, were found to be absent during mCMV latency in the lungs. *Objectives:* We will here review recent data on the variegated expression of IE-phase genes *ie1* and *ie2* during mCMV latency in the lungs, and on the expression patterns found in transcriptional foci during induced reactivation. We will discuss immunological implications of *ie1* gene expression during latency and will speculate a bit on how CD8 T cells might trigger latency-associated *ie1* gene expression in a regulatory circuit.

Keywords: Cytomegalovirus; Enhancer; Immediate-early genes; Latency; Reactivation; Recurrence

1. In essence, latency means...

The definition of viral latency implies that functional viral genome is retained in the host after resolution of productive primary infection, and that certain signaling events can reactivate productive cycle gene expression, which, in particular in an immunocompromised host, results in recurrence of infectious virus and recrudescence of clinical symptoms (Roizman and Sears, 1987).

Fig. 1 sketches the principal course of CMV infections. Primary infection is associated with

Abbreviations: BMT, bone marrow transplantation; hCMV, human cytomegalovirus; IE, immediate early; mCMV, murine cytomegalovirus; MHC, major histocompatibility complex; NK, natural killer; PFU, plaque forming unit; RT PCR, reverse transcriptase polymerase chain reaction; TCR, T cell receptor; TNF, tumor necrosis factor.

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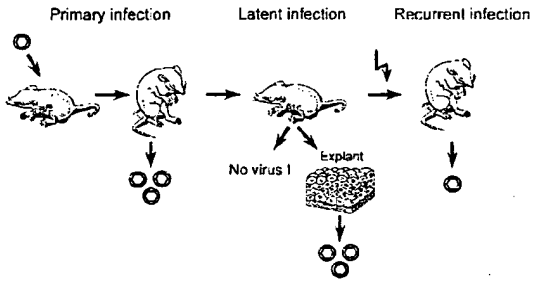

Fig. 1. Definition of latency.

virus replication in various types of tissues at multiple organ sites. The extent of virus production and the development of CMV disease, however, depend largely on the immune status of the host. In immunocompetent mice, mCMV replication is prominent only in the salivary glands, while many organ sites are affected in the immunocompromised host, including the lungs, spleen stroma, liver, heart, bone marrow stroma, gastrointestinal tract, glomeruli in the kidney cortex, suprarenal glands, and the choroid plexus and ventricular ependyma of the brain (evidence from many publications; for a more recent work, see Podlech et al., 1998). Accordingly, many different cell types are infected in vivo by mCMV, including various types of epithelial cells (for instance: glandular epithelial cells of the salivary glands, pneumocytes, enterocytes, and hepatocytes) as well as endothelial cells, myocytes, brown fat adipocytes, connective tissue fibrocytes, bone marrow stromal cells, dendritic cells, and tissue macrophages. In our studies, B- and T-cell compartments of lymphoid organs, including the thymus, were found to be not infected.

After clearance of the productive infection, which is effected at most sites primarily by antiviral CD8 T cells (Reddehase et al., 1985; Podlech et al., 1998, 2000), latency is established (for a review, see Jordan, 1983). During latency, by definition, infectious virions are undetectable with direct infectivity assays, such as the plaque assay in which permissive cells, usually fetal fibroblasts grown as monolayers in cell culture, are inoculated with tissue homogenate. That viral genome was not cleared, but instead was still present in the host, became evident from the in vivo recurrence of the productive infection upon immunoablative treatment of latently infected mice with anti-lymphocyte serum (Gardner et al., 1974), with a combination of anti-lymphocyte serum and corticosteroids (Jordan et al., 1977) or with cyclophosphamide (Mayo et al., 1977). Because recurrent virus became first detectable in salivary glands, these were considered as a likely site of latency. The overall viral load during recurrence is usually lower than during primary infection, since antibodies, even though they cannot prevent virus reactivation, limit the dissemination of recurrent virus (Jonjic et al., 1994; Reddehase et al., 1994). Early evidence for additional organ sites of latency and recurrence was provided by the demonstration of virus recurrence in tissue explant cultures. Specifically, Cheung and Lang (1977) reported virus recurrence in salivary gland as well as in prostate explant cultures, and Jordan and Mar (1982) established the spleen explant technique, which proved to reactivate mCMV most reproducibly. Later, Wilson et al. (1985) reported recurrence in cardiac explants. In accordance with these findings in tissue cultures, transplantation of organs from latently infected donors resulted in virus recurrence within the transplant and in subsequent disseminated disease in immunosuppressed recipients (Hamilton and Seaworth, 1985; Schmader et al., 1995).

2. 'Low-level persistence' versus 'molecular latency': a dead heat for more than a decade

Absence of infectivity turned out to be a soft criterion for the definition of latency, as it depends on the sensitivity of assays available for the detection of infectious virions. As a consequence, it had long remained an open question whether viral genome is maintained during latency in absence of virus production (Molecular Latency Hypothesis) or whether virus production persists on a low level, namely below the detection limit of infectivity assays (Low-level Persistence Hypothesis). Apparently, absence of infectivity, if observed with limited assay sensitivity, cannot prove molecular latency and cannot disprove low-level persistence.

The strongest argument in favor of persistent productive infection was adduced by the group of J. Stevens (Yuhasz et al., 1994) who detected IE1 transcripts in absence of detectable virus in the lungs of latently infected mice. The authors concluded that this transcript, at that time generally regarded as a productive cycle transcript, indicated productive infection with a sensitivity superior to the sensitivity of the then available infectivity assays. As we will discuss below, this view needs to be revised.

More recently, highly improved assays for the detection of infectivity indicated a true molecular latency of mCMV in spleen and kidney (Pollock and Virgin IV, 1995) as well as in the lungs (Kurz et al., 1997). For defining assay sensitivity on a molecular level, it is important to determine the genome-to-infectivity ratio, that is the number of viral genomes required for initiating productive infection. Obviously, this ratio can not fall below 1:1, since a minimum of 1 genome is required for infection. However, the experimental ratio is expected to be $> 1:1$, because defective genomes, defective particles, or particles disruptured during the preparation are likely to exist. In addition, multicapsid virions are formed during mCMV morphogenesis in most cell types, except in glandular epithelial cells of the salivary glands. Kurz et al. (1997) have analyzed purified cell-culture derived mCMV preparations by electron microscopy, and have found a monocapsid-to-multicapsid virion ratio of approximately 3:2, with multicapsid virions harboring 3.4 (range, 2–8) capsids on average. Taking into account also disruptured particles, a minimum genome-to-infectivity ratio of 2.6:1 was predicted. By contrast, direct Southern blot analysis showed that 1 plaque forming unit (PFU; measured on fetal fibroblasts) contains approximately 500 viral DNA molecules, which at first glance suggested a very high rate of non-infectious particles. That this is not the case was demonstrated by the experiment summarized in Fig. 2 (based on data from Kurz et al., 1997). Homogenate of latently infected lungs (an aliquot containing 20 000 copies of latent viral DNA) was tested for infectivity by inoculating a fetal fibroblast monolayer under conditions of centrifugal enhancement of virion penetration and infectivity (Hudson et al., 1976). Since plaques did not develop, polyadenylated RNA was isolated from the inoculated indicator cells and tested by RT PCR for the presence of IE1 transcripts. The negative result led to the conclusion that the viral DNA present in the inoculum represented latent viral genomes not contained within infectious particles. Further aliquots of the lung homogenate were substituted with defined amounts of purified virus, titrated from 100 PFU to 0.01 PFU per culture. The centrifugal enhancement led to a 20-fold increase in infectivity, leading to two plaques (on average) for an inoculum dose of 0.1 PFU. With the enhanced sensitivity of an IE1-specific RT PCR, a replication-dependent (time-dependent and phosphonoacetic-acid sensitive) signal was detected for an inoculum dose of just 0.01 PFU, which corresponds to 5 viral DNA molecules. A statistical analysis (for details, see Kurz et al., 1997) revealed a genome-to-infectivity ratio of 4.5 (95% confidence limits 2.0–9.0). Since the estimated minimum of 2.6 genomes lies within the confidence limits of the experimentally determined value, the maximum of the assay sensitivity was reached.

In conclusion, (i) non-infectious particles are not frequent in mCMV, (ii) the assay can detect infectivity with an optimal sensitivity, and (iii) viral genomes present in latently infected lungs are not in an infectious state.

3. Sites of latency: a matter of controversy

The early studies on virus reactivation in explant cultures and by organ transplantation (see above) had already indicated the presence of latent mCMV in various organs. Accordingly, more recent studies using PCR have demonstrated the presence of viral DNA in many organs of latently infected mice (Klotman et al., 1990; Balthesen et al., 1993, 1994a; Collins et al., 1993; Reddehase et al., 1994), including salivary glands, lungs, spleen, heart, suprarenal (adrenal) glands, and kidney. Viral DNA present in intravascular leukocytes could easily have explained the detection at multiple organ sites. Absence of viral DNA in blood at the time of the assay was therefore crucial for the

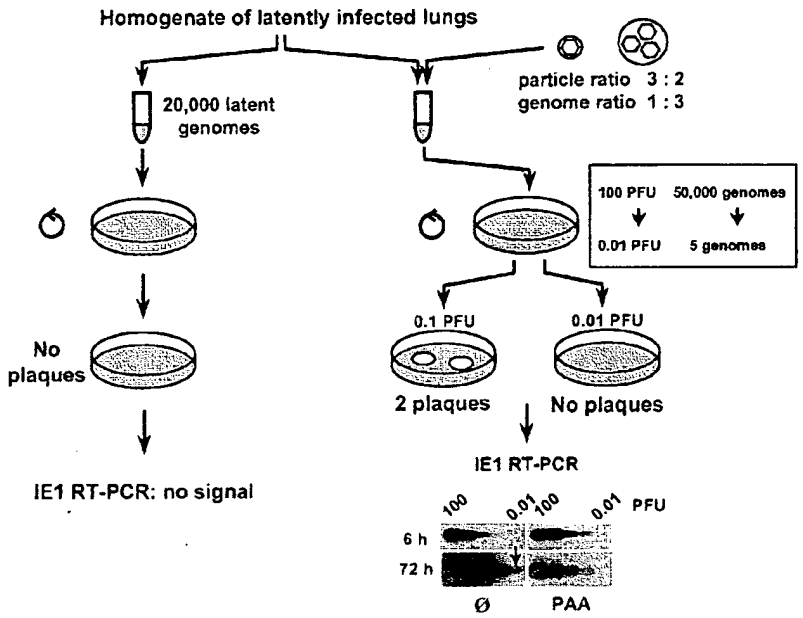

Fig. 2. Evidence for molecular latency of mCMV in the lungs. The genome-to-infectivity ratio for mCMV was determined by centrifugal infection of fetal fibroblast monolayers with an aliquot of lung homogenate from latently infected mice (left) or with an aliquot that was substituted with defined amounts (titrated from 100 to 0.01 PFU, corresponding to 50 000 to 5 genomes, respectively) of purified virions (right). An RT PCR specific for IE1 was performed with polyadenylated RNA isolated from the cultures. Shown are the autoradiographs (original data from Kurz et al., 1997) obtained after Southern blot and hybridization with an internal probe. PAA, infection performed in the presence of phosphonoacetic acid.

conclusion that viral latency can be localized in stromal and/or parenchymal cells of the respective organs (Balthesen et al., 1993, 1994a).

That cells of the myeloid hematopoietic lineage, specifically macrophages/dendritic cells and their progenitors, are among the cellular sites of mCMV latency dates back to a study by Brautigam et al. (1979) and was supported by more recent work (Mitchell et al. 1996; Pollock et al., 1997). By contrast, Mercer et al. (1988) localized latent viral DNA to an MHC class-II-negative cell of the stromal compartment of the spleen, putatively the endothelial-like sinusoidal lining cell. Likewise, Pomeroy et al. (1991) detected latent viral DNA in the stroma of the spleen. Renal peritubular epithelial cells were discussed as the cellular site of latency in the kidney (Klotman et al., 1990). Accordingly, in situ PCR suggested the existence of more than one cellular site, specifically histiocytes and endothelial-like cells (Koffron et al., 1998).

Our own studies (summarized in Fig. 3) demonstrated that the localization of viral DNA is highly influenced by the clearance kinetics after primary infection. Different time points of the analyses as well as different experimental protocols, including different immune status of the mice at the time of primary infection, different routes of inoculation and variance between virus isolates, may thus explain controversial findings in the literature. In a model of CMV disease after primary infection of immunocompromised bone marrow transplantation (BMT) recipients with purified, cell culture-propagated mCMV strain Smith ATCC VR-194/1981, productive infection was found to be resolved first in bone marrow and later in organs, with a hierarchy of spleen being cleared faster than lungs and lungs faster than salivary glands. Viral

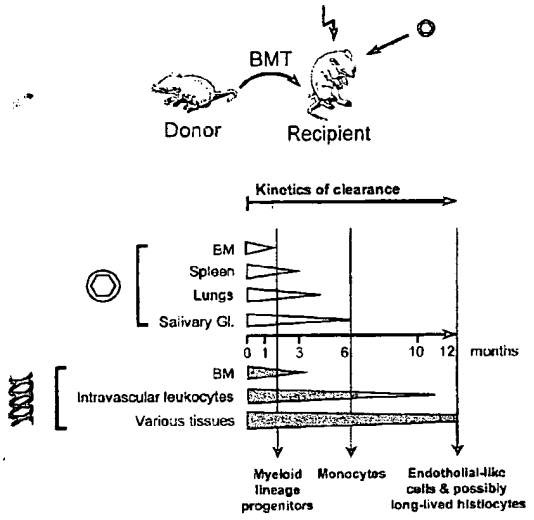

Fig. 3. Multiple sites of latency. The clearance of infectious virus (virus symbol) and of viral DNA (helix symbol) was measured in a model of syngeneic bone marrow transplantation (BMT) with BALB/c mice as bone marrow (BM) cell donors and recipients. Enduring latency is established in many organs (including salivary glands, spleen, lung, heart, kidneys, and suprarenal glands) after clearance of viral DNA from the BM and from the intravascular compartment.

DNA was also cleared first from the bone marrow and much later from intravascular leukocytes, while it was retained for the life span of the recipients in multiple organs (Balthesen et al., 1994b; Reddehase et al., 1994; Kurz et al., 1997, 1999). As shown by Mitchell et al. (1996), mCMV DNA present in blood during acute infection is primarily found in monocytes and in polymorphonuclear leukocytes. This DNA most likely was picked up from productively infected tissue cells and vascular endothelium.

Studies on hCMV latency have led to the hypothesis that latently infected myeloid progenitor cells in the bone marrow are the source of latent virus at extramedullary sites (Kondo et al., 1994; Hahn et al., 1998; Slobedman and Mocarski, 1999). While mCMV DNA is also detectable in bone marrow cells, the kinetics of viral DNA clearance (see above) excludes the bone marrow as the source of latent mCMV in tissues. At any time point during latency, the relative load of viral DNA at extramedullary sites was much higher than the relative load in bone marrow. One might argue that a highly expanded progeny of few latently infected myeloid progenitor cells can account for a high absolute load of latent viral DNA. However, one has to consider the fact that the uninfected progenitor cells likewise generate a highly expanded progeny. Thus, unless we assume that latently infected progeny expand to a larger clone size or that infected progeny have a prolonged life span, for instance by an anti-apoptotic function of latency-associated gene expression, we cannot explain an extramedullary enrichment of latent viral DNA (Balthesen et al., 1994b).

That mCMV and hCMV may differ in this respect is an often heard argument. It is undoubted that hCMV DNA and latency-associated transcripts are present in a low proportion of bone marrow myeloid progenitor cells in asymptomatically infected persons. However, whether these cells account for the extramedullary latency is a different question. The load comparisons and the clearance kinetics still need to be performed for hCMV. Admittedly, this is extremely difficult if not impossible to do, as it would require a longitudinal comparative analysis of viral DNA load in blood leukocytes and organ biopsies, for instance in BMT patients who have undergone primary hCMV infection. Nevertheless, without that clinical data, there is at present no reason to doubt the message given by the mouse model.

4. Load and risk

Murine models have helped to document that the risk of recurrent CMV infection is dependent upon the load of latent viral DNA in tissues, and that the conditions of primary infection predetermine the risk of recurrence by modulating the load (Reddehase et al., 1994). Specifically, low virus replication during primary infection of immunocompetent, adult mice led to a low load of latent viral DNA in organs, and was associated with a low incidence of recurrence after immunoablative treatment. By contrast, prolonged high-level virus replication during primary infection of neonatal mice led to a high load of latent viral DNA and

was associated with a high incidence of recurrence after immunoablative treatment. Notably, the higher risk of recurrence in mice infected as neonates was observed in spite of a higher titer of neutralizing serum antibody. That antibodies do not prevent recurrence but limit the spread of recurrent virus was documented by comparing recurrence in normal μ+/μ+ and B-cell deficient μ−/μ− mice (Jonjic et al., 1994). Inhibition of hematogenic virus dissemination in infected mice by antiviral antibody was demonstrated by transfer of serum derived from immune μ+/μ+ mice, using serum from immune μ−/μ− donors in the control experiment (Reddehase et al., 1994). The medically important conclusion was that the risk of recurrence depends on the time-point of primary infection (perinatal phase > day-care center age > adult age), and that the viral DNA load is a better predictor of risk than is seropositivity alone.

Organ-specific differences in the viral load correlated with recurrence incidences in the respective organs. Specifically, lungs proved to be a major site of mCMV latency and recurrence, a finding that may relate to the fact that interstitial pneumonia is a frequent manifestation of CMV disease (Balthesen et al., 1993). While the overall virus replication and dissemination in the host is important for the establishment of latency at multiple sites, the load of latent viral DNA in a particular organ does not always correlate with the amount of virus production in that organ (Balthesen et al., 1994a; Reddehase et al., 1994). For instance, after infection of adult and immunocompetent mice, the latent virus load in the lungs was higher than in the salivary glands, even though productivity during acute infection was confined to the salivary glands (Reddehase et al., 1994). Productive CMV infection is usually cytolytic. Accordingly, cells which account for the bulk of virus production during acute infection are not necessarily those in which latency is established. It is reasonable to propose an analogy to α-herpesvirus infection, where productive infection occurs in epithelial cells, while latency is established in neurons. As discussed above, the latently infected cell type(s) is not yet unequivocally identified for CMVs.

Therapeutic intervention that reduces the load of latent viral DNA can reduce the risk of recurrence. This has been demonstrated experimentally in a model of syngeneic BMT and adoptive antiviral cytoimmunotherapy of acute mCMV infection with antiviral CD8 T cells (Fig. 4; derived from Steffens et al., 1998). Notably, by controlling acute infection, CD8 T cells reduced the subsequent load of latent viral DNA in the lungs from approximately 5,000 copies per million cells to approximately 1,000 copies per million cells, but even extremely high doses of CD8 T cells could not prevent the establishment of latency. These data indicated the existence of two types of latency, one being susceptible and the other one being resistant to control by CD8 T cells. Whether this finding is explained by two different latently infected cell types is unknown. Importantly, prevention of the CD8 T-cell susceptible component proved to be biologically meaningful in that the high load and the low load condition were associated with high and low incidence of recurrence after secondary immunoablative treatment, respectively.

5. Mechanism of recurrence: withdrawal of immune control as the common trait

Different modes of inducing recurrence in latently infected mice have been described: treatment with anti-lymphocyte serum and corticosteroids (Gardner et al., 1974; Jordan et al., 1977), with cyclophosphamide (Mayo et al., 1977), or genotoxic stress by γ-irradiation (Balthesen et al., 1993; Reddehase et al., 1994). There is an early report indicating that pregnancy induces reactivation (Baskar et al., 1985), and data presented at this meeting by Cook et al. (C.H. Cook, pers. comm., 2001) showed that sepsis can induce recurrence. While all these different treatments also involved signaling by cytokines, release of latent virus from immune control appears to be a common trait. The same applies to tissue explantation and transplantation, which both involve stress conditions for the cells as well as withdrawal of immune cells. The most direct approach to investigate the role of immune cells in the control

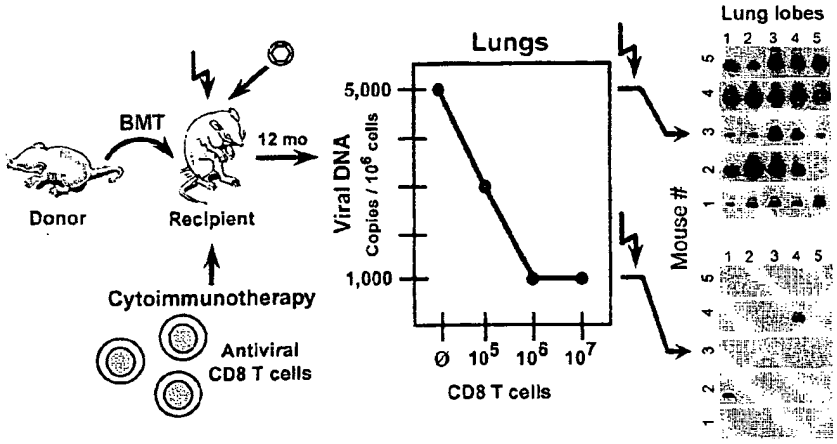

Fig. 4. Latent viral DNA load and risk of recurrence: intervention by cytoimmunotherapy. Acute infection in BMT recipients was treated by adoptive transfer of antiviral CD8 T cells. Control of productive infection also resulted in a lower load of latent viral DNA and in a lower incidence of recurrence after secondary hematoablative treatment (original data from Steffens et al., 1998). Recurrence was measured by infection of fetal fibroblasts with homogenates (see Fig. 2) from the five lobes of the lungs. Shown are the autoradiographs obtained after IE1-specific RT PCR, Southern blot of the amplificates, and hybridization with an internal probe.

of latency was the selective in vivo elimination of cell subsets in B-cell deficient $\mu-/\mu-$ mice (Polic et al., 1998). In this experimental system, the absence of antibody facilitated virus multiplication and dissemination after recurrence, which increased the sensitivity of detection. On the other hand, dissemination of the recurrent virus made it more difficult to localize the site of recurrence. In essence, the work documented a hierarchical and redundant immune control of latency, with contributions made by CD8 T cells, CD4 T cells, and natural killer (NK) cells. Combined depletion of all three types of lymphocytes resulted in the highest observed incidence of recurrence. The authors concluded that CMVs differ from α-herpesviruses in that recurrence does not require a molecular activation of productive gene expression by cytokine signaling, but results from withdrawal of the controlling immune cells.

However, as with all other modes of the induction of recurrence discussed above, depletion of immune cells is not just a subtraction of a specific cell type but is accompanied by a storm of cytokine-mediated signaling that can molecularly activate the productive viral cycle. We have to consider the problem that read-out of infectious virus as the end-point of reactivation does not allow us to distinguish between molecular reactivation of productive gene expression and the subsequent multiplication and dissemination of recurrent virus.

In conclusion, immune cells, and primarily CD8 T cells, control CMV recurrence. Whether these cells control also molecular reactivation is a different question not yet answered.

6. Selective generation of IE1 transcripts during mCMV latency in the lungs

The definition of latency does not imply that the viral genome is transcriptionally silent. The highly regulated transcriptional program can be interrupted at many checkpoints before the assembly and release of infectious virions. From our knowledge of productive infection, it seems to be clear that the regulation of IE gene expression is an early (and possibly the first) checkpoint on the way from latency to recurrence. In mCMV, the IE1/3 transcription unit (corresponds to IE1/2 in hCMV) consists of exons 1–5 and gives rise to IE1 (exons 1–4) and IE3 (exons 1–3 plus 5) mRNAs by differential splicing (Keil et al., 1987; Messerle et al., 1992). The IE1/3 transcription unit is driven by

promoter $P^{1/3}$ that is controlled by a strong upstream enhancer (Dorsch-Häsler et al., 1985). The enhancer serves as a molecular switch that links the IE1/3 transcription to the microenvironment of the (latently) infected cell. External stimuli, e.g. pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α produced by macrophages and activated type-I T cells, induce transcription factors, which activate the enhancer by binding to defined sequence motifs. It was previously thought that IE1 were an essential transactivator protein and that IE1 mRNA could therefore be used as a marker for productive infection. Accordingly, detection of IE1 transcripts in latently infected spleens (Henry and Hamilton, 1993) and lungs (Yuhasz et al., 1994) was taken for an evidence for recurrent or chronic infection.

The role of IE1 in acute infection was revised recently. That protein IE3 instead of IE1 is the key transactivator of early gene expression was concluded from reporter gene assays by Messerle et al. (1992), and an essential role for IE3 was proven by replication-deficiency of an *ie3* gene (exon 5 of *ie1/3*) deletion mutant of mCMV (Angulo et al., 2000). In contrast, IE1 is a non-essential co-transactivator of early gene expression. Own recent work on mCMV latency in the lungs (Fig. 5; modified from Kurz et al., 1999) showed an expression of IE1 mRNA, whereas IE3 mRNA, downstream transcripts such as of *gB*, and infectious virions were undetectable. This work thus gave the first evidence for a control of mCMV latency after IE1/3 transcription initiation, putatively at the step of IE1/3 precursor splicing or splice product stabilization. Notably, recent work by Hummel et al. (2001) showed that this second checkpoint cannot be overruled by cytokine (TNF-α)-enhanced generation of IE1 transcripts.

7. Latency-associated IE1 transcription in the lungs shows variegation

Variegated (mosaic) expression caused by silencing and de-silencing is a regular feature of gene expression often seen with transgenes (for a review, see Fiering et al., 2000). Notably, when Kurz et al. (1999) studied *ie1* gene expression in pieces of latently infected lungs, a random on-or-off pattern was observed (Fig. 6, left panel). It should be noted that latent viral DNA was evenly distributed between the pieces, with each piece harboring approximately 20 000 viral genomes. Fig. 6, center panel, illustrates the variegated

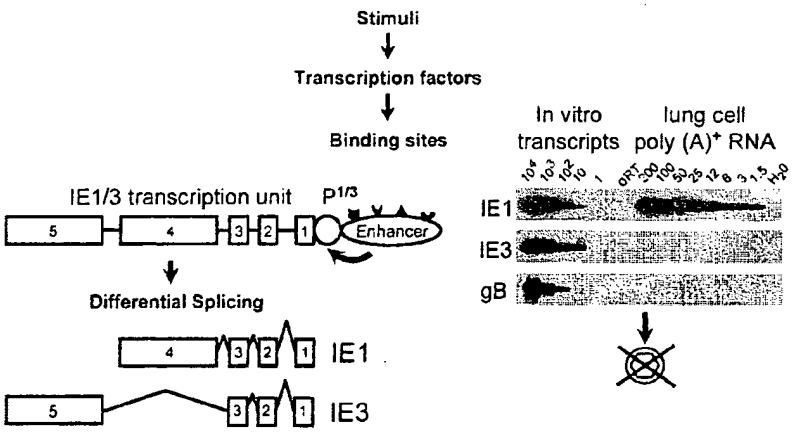

Fig. 5. Control of latency after IE1/3 transcription initiation. Left, map of the IE1/3 transcription unit controlled by promoter $P^{1/3}$ and an upstream enhancer. IE1 (exons 1–4) and IE3 (exons 1–3, and 5) transcripts are generated by differential splicing. Right, selective generation of IE1 transcripts in latently infected lungs (original data from Kurz et al., 1999). Shown are autoradiographs obtained after specific RT PCRs, Southern blot of the amplificates, and hybridization with the respective internal probes.

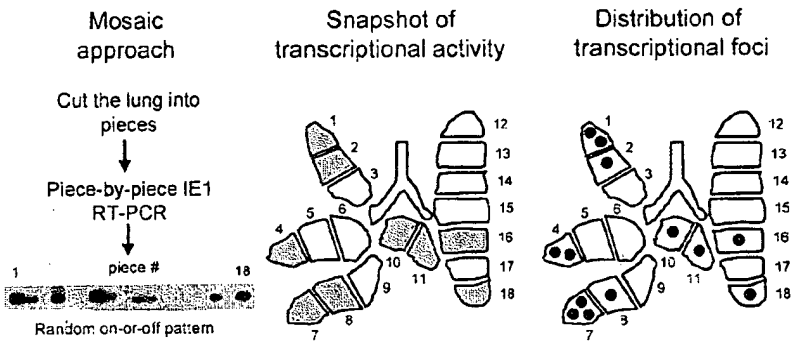

Fig. 6. Variegated expression of IE1 during latency in the lungs. Left, mosaic approach and results of a piece-by-piece IE1-specific RT PCR, revealing a random on-or-off pattern of IE1 transcription. Center, topographical map illustrating the variegated expression. Right, frequency and distribution of transcriptional foci obtained by applying the Poisson distribution function.

pattern of transcriptionally silent and active pieces as a topographical map representing a snapshot of expression. The analysis of individual mice revealed random patterns, and we propose that pieces would switch randomly from active to silent and vice versa in a time-lapse simulation. It is important to understand that a positive piece must include at least one transcriptional event, referred to as a focus of transcription, but may contain also two or more foci. The Poisson law (see Appendix of the work by Grzimek et al., 2001) allows us to calculate the fractions of pieces containing one or two or more foci (Fig. 6, right panel). The statistical analysis gave an estimate of 1 IE1 transcriptional focus per approximately 5 million lung cells. Based on the viral DNA load of approximately 5,000 copies per million cells, this equals 1 focus per approximately 25 000 latent viral genomes. In essence, at any time point, the vast majority of latent viral genomes in the lungs is silent for IE1 expression, and variegated expression of IE1 represents very rare events.

8. Random, asynchronous, and asymmetric transcriptional activity of the enhancer-flanking genes *ie1* and *ie2*

In mCMV, the enhancer governs two cognate genes: *ie1/3* (see above) and *ie2* (Messerle et al., 1991). Gene *ie2* possesses its own promoter, P^2, and is transcribed in a direction opposite to *ie1/3*. It has no counterpart in hCMV, and the mCMV IE2 protein is dispensable for virus growth in cell culture and in vivo (Manning et al., 1988; Cardin et al., 1995). If the variegated expression of *ie1* were the result of random 'noise signaling' to the enhancer by cytokine-induced transcription factors, *ie1/3* and *ie2* should be transcribed synchronously, that is one should find 'on–on' and 'off–off' patterns of transcription in pieces of the lungs. While random, variegated transcription of *ie2* was indeed found, the observed patterns were 'on off' and 'off–on' in a ratio of 2:1 (Grzimek et al., 2001). Apparently, the enhancer did not synchronize transcription from its two cognate genes. This may indicate either that the enhancer operates asymmetrically by its nature, for example due to the structural asymmetry in the distribution of transcription factor binding sites, or that the enhancer is not involved at all in the variegated expression during latency. One alternative mechanism could be that an open chromatin-like structure develops randomly around $P^{1/3}$ and P^2 in latent viral episomes. Since the distance between the two promoters is 1,375 bp, which equals the distance of 7 nucleosomes in cellular DNA, the chromatin-like structure may simultaneously be open at one end and closed at the other end of the $P^{1/3}$-E-P^2 regulatory unit.

9. Variegated IE gene expression is driven by a self-perpetuating domino effect: a working hypothesis

What could be the driving force for the random expression of *ie1* (and *ie2*) in latently infected lungs? There is good evidence for *ie1* gene expression after activation of the enhancer via the TNF-α/NF-κB and AP-1 signaling pathways (Prösch et al., 1995; Hummel et al., 2001). Specifically, Hummel et al. (2001) found that transplantation of latently infected kidneys to allogeneic recipients results in elevated IE1 transcription, but not in reactivation of the productive cycle. TNF-α substituted for an allogeneic immune reaction only in the lungs, and, at this particular site, stimulation of the enhancer by TNF-α appeared to involve the transcription factors NF-κB and AP-1.

Likewise, 'noise' cytokine (e. g. TNF-α) signals in latently infected lungs could account for the sporadic IE1 transcription. However, there is another possibility (Fig. 7). Activated CD8 T cells are known to secrete TNF-α. We can therefore propose the following scenario (for BALB/c mice, haplotype H-2^d): a cytokine-mediated signaling activates the enhancer in a latently infected cell. This leads to IE1 transcription, protein synthesis, antigen processing, and presentation of the immunodominant IE1 peptide YPHFMPTNL (Reddehase et al., 1989; Holtappels et al., 2000). IE1-specific memory CD8 T cells (CD62L-high expressors) become activated (CD62L-low expressors) and secrete TNF-α, which turns on the IE1 expression in another latently infected cell. In support of this hypothesis, recent publications have shown that protective, interstitial memory CD8 T cells persist in the lungs after clearance of productive infection (Podlech et al., 2000), and that CD8 T cells specific for the IE1 peptide are enriched during latency in the lungs in a CD62L-low subpopulation (Holtappels et al., 2000).

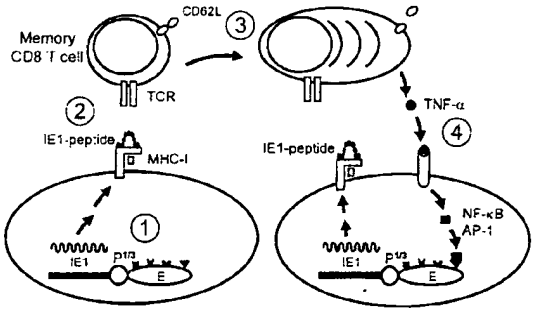

Fig. 7. Activated CD8 T cells induce IE1 expression in a regulatory circuit: a hypothesis. Step 1, an assumed signal activates the enhancer, E, of the viral genome that is present in a latently infected cell. This results in IE1 transcription and protein synthesis. Step 2, as a result of IE1 protein processing, the immunodominant IE1 peptide is presented, and the peptide-MHC class-I complex is recognized by the T cell receptor (TCR) of an IE1-specific memory CD8 T cell. Step 3, the memory cell becomes activated (the L-selectin CD62L is cleaved proteolytically upon activation) and cytokines, specifically TNF-α, are secreted. Step 4, TNF-α binds to TNF-receptor(s) on a second latently infected cell and induces transcription factors, which bind to and thereby activate the enhancer; and so forth.

Whether the IE1-expessing latently infected cells are killed in vivo is an open question. The finding that the latent viral DNA is not eventually cleared from the lungs may argue against cytolysis. However, the rate of the proposed killing is an unknown parameter. The frequency of the expression is low, the copy number of the latent viral genome per cell is unknown, and we cannot exclude the possibility that the latent viral DNA can be replenished by cell division.

10. Variegation of viral gene expression during reactivation

Previous studies on virus reactivation and recurrence have considered only the end-point of reactivation, that is the recurrence of infectious virus, and have thus not distinguished between molecular reactivation and recurrence. So far there is only one study on viral gene expression during reactivation (Kurz and Reddehase, 1999). The mosaic approach (introduced in Fig. 6) was applied here again (Fig. 8). Latently infected mice received hematoablative, immunosuppressive treatment by total body γ-irradiation, and reactivation of viral gene expression as well as recurrence of infectious virus were analyzed in pieces of the lungs for groups of mice at days 4, 8, and 12. Surprisingly, the phenomenon of variegation was again observed. Specifically, there still existed

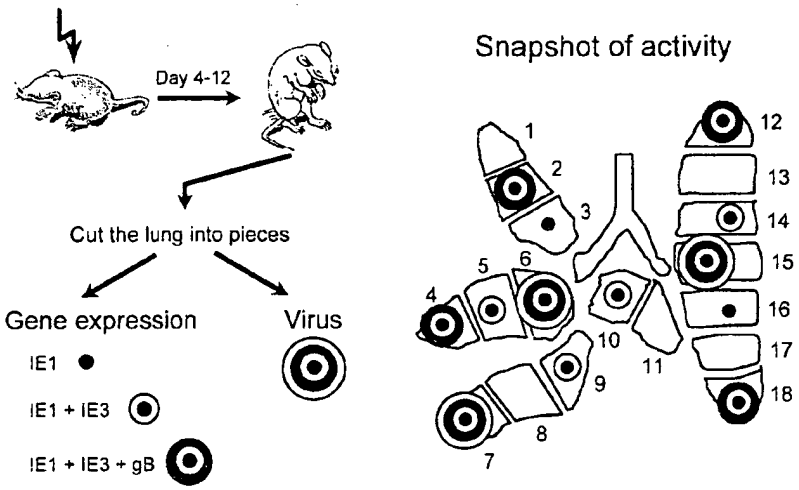

Fig. 8. Variegated expression of viral genes during reactivation and recurrence. Latently infected mice were subjected to hematoablative treatment by γ-irradiation, and viral gene expression as well as virus recurrence in the lungs were measured according to the mosaic approach by a piece-by-piece analysis. Some pieces had remained transcriptionally silent and some still displayed the latency phenotype of IE1 expression. In other pieces, however, the transcriptional program had proceeded to IE3, to gB, or to the generation of infectious virus. Note that a positive piece can harbor more than one focus and that foci of lower rank (IE1 < IE3 < gB < virus) are hidden behind foci of higher rank. The number and distribution of all foci can be calculated by applying the Poisson distribution function (result shown in Fig. 9, right).

pieces containing 20000 transcriptionally silent viral genomes. Other pieces still displayed the 'latency phenotype' of IE1 expression in absence of IE3. However, some pieces had proceeded to spliced IE3 mRNA, some to gB mRNA, and a few had made all the way through to the release of infectious virus.

It had previously been postulated that latent mCMV reactivates spontaneously, and that maintenance of latency is entirely based on immune control (Polic et al., 1998). In that case, withdrawal of immune control should lead to an accumulation of recurrence events with time. Notably, the observed patterns did not shift to more events of recurrence between days 4 and 12. It was therefore concluded that γ-irradiation, in addition to its immunosuppressive effect, has an inductive function. One may speculate that repair mechanisms induced in the cells in response to the genotoxic stress involve splice control proteins that overrule the block in IE3 splicing.

11. Viral gene expression during reactivation indicates the existence of multiple, sequentially ordered checkpoints in the transition from mCMV latency to recurrence

The reactivation data have also shown that viral transcription remains arrested in most foci at a stage before recurrence. Kurz and Reddehase (1999) proposed a model of multistep virus reactivation involving multiple checkpoints (Fig. 9). A signal 1 is required for activating the enhancer to initiate IE1/3 transcription. One candidate for signal 1 is TNF-α, but it is likely that many more signals operate via transcription factor binding to the enhancer. A signal 2 is required to overrule the block in IE3 splicing, but even the generation of the essential transactivator protein IE3 does not inevitably lead to progression of the productive cycle. Thus, the existence of further checkpoints has to be postulated. That latency is characterized by a stop at the second checkpoint

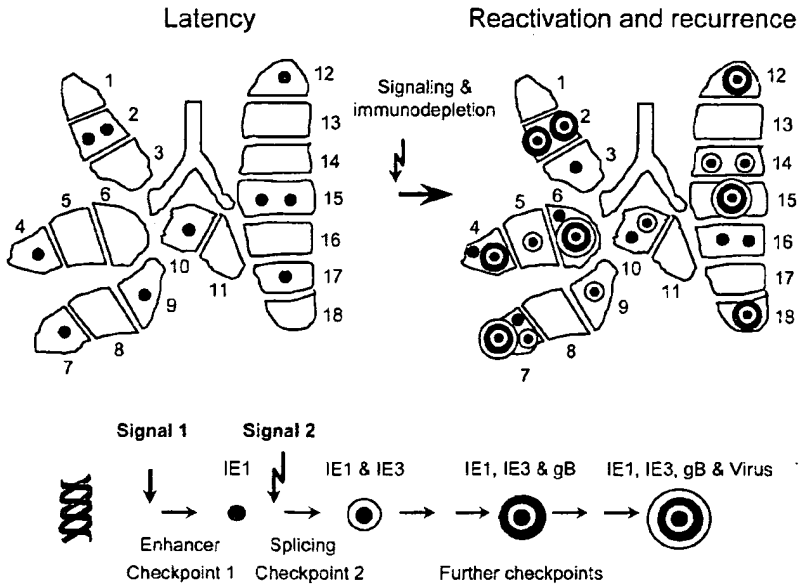

Fig. 9. Summary: multistep model of CMV reactivation and recurrence (modified from Kurz and Reddehase, 1999). Left, statistically generated prototypic lung during latency. IE1 is expressed in a random manner. Note that IE2 is also expressed randomly (not depicted), whereas transactivator IE3 is absent. Right, statistically generated prototypic lung during reactivation and recurrence.

applies to the lungs. Work by Mitchell and Kercher presented at this meeting (B.M. Mitchell, pers. comm., 2001) suggests that in the specific signaling environment of ocular tissue, viral gene expression during latency is arrested after gH.

Strikingly, the frequency of transcriptionally active foci was not dramatically increased after induction by γ-irradiation, namely just by a factor of 2. This result is compatible with the hypothesis that IE1 foci, which in absence of signal 2 would return to quiescence, move on in the replicative cycle to the next checkpoint(s), whereas new IE1 foci are generated by the same mechanism that generates IE1 foci during latency.

12. Conclusion

The possibility to study CMV latency and reactivation in vivo is the main advantage of animal models. The mouse model has shown that viral latency and reactivation are controlled not only at the major IE promoter–enhancer, but that there exist further molecular checkpoints downstream in the program of productive cycle gene expression. One checkpoint is effective after IE1/3 transcription initiation and prevents splicing of the essential IE3. This block cannot be overruled by enhanced IE1/3 transcription. Genes *ie1* and *ie2* are transcribed during latency in the lungs in a random fashion from a very low number of the latent viral genomes. There is evidence to suggest that expression of gene *ie1* results in presentation of the antigenic IE1 peptide and, as a consequence, in an activation of tissue-resident memory CD8 T cells. The role of T cells can be 2-fold: T cells may be involved in the control of latency by eliminating IE1 peptide-presenting cells. On the other hand, cytokines secreted by activated T cells may stimulate *ie1* gene expression in latently infected, transcriptionally silent cells by signaling at the enhancer. It appears to be established that immune cells, CD8 T cells in particular, control latency by preventing virus multiplication. Whether there is a

role of immune cells in the molecular control of transcriptional reactivation is a focus of current research.

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